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# Sensing crop nitrogen status with fluorescence indicators. A review

Nicolas Tremblay · Zhijie Wang · Zoran G. Cerovic

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**Abstract** The optimization of nitrogen (N) fertilization is the object of intense research efforts around the world. Overfertilization is commonly used as a form of insurance against uncertain soil fertility level. However, this practice results in lower nitrogen use efficiency, high levels of residual N after harvest, and losses in the environment. Determining an N recommendation that would preserve actual crop requirements, profitability of the farm, and quality of the environment has been subjected to a number of research initiatives with a variable degree of success. On one hand, soil tests are capable of estimating the intensity of N release at any point in time, but rarely the capacity factor over a longer period. On the other hand, in the context of in-season N applications, crops are often considered good integrators of factors such as the presence of mineral N, climatic conditions, soil properties, and crop management. Strategies have been proposed with plant sensor-based diagnostic information for N recommendations, but the sensitivity of reflectance-based parameters alone do not provide complete satisfaction (delayed

sensitivity, need of specific chlorophyll, biomass or cover fraction ranges, lack of specificity to the N stress). Fluorescence sensing methods have been used to monitor crop physiology for years, and they may offer solutions for N status diagnosis over reflectance-based methods. In this paper, we review three plant fluorescence components related to four sensing approaches—variable chlorophyll fluorescence, leaf chlorophyll content-related fluorescence emission ratio, blue-green fluorescence, and epidermal screening of chlorophyll fluorescence by phenolic compounds—from the perspective of their relevance to N fertilization management of agricultural crops. We examine the existence of N-induced changes in each case, together with applications and limitations of the approach. Among these approaches, the fluorescence emission ratio method is the most important and the most widely used to date. However, blue-green fluorescence and epidermal screening of chlorophyll fluorescence by phenolic compounds has also received a great deal of attention particularly with the recent commercial release of instruments which can measure in real time and in vivo both the leaf chlorophyll content and several phenolic compounds (anthocyanins, flavonoids, hydroxycinnamic acids). Overall, our conclusion is that fluorescence-based technologies allow for highly sensitive plant N status information, independently from soil interference, leaf area, or biomass status. They also allow for probing not only the chlorophyll status but also other physiological parameters known to react to N fertility conditions. These new parameters have the potential to provide new N status indicators that can be assessed remotely in a precision agriculture context.

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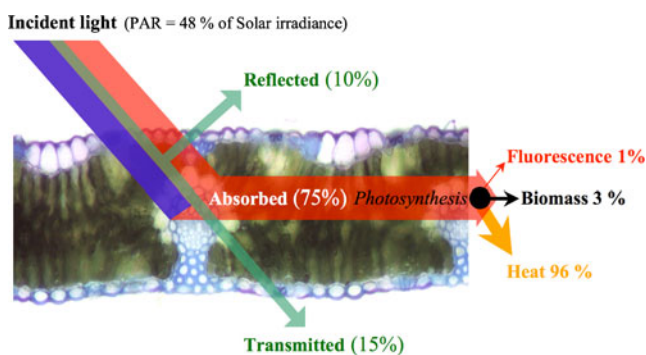
**Keywords** Chlorophyll fluorescence · UV-excited fluorescence · Optical sensors · Nitrogen status · Precision crop management · Polyphenolics

## Contents

1. Introduction . . . . .	2
2. Plant fluorescence components. . . . .	2
2.1. Chlorophyll fluorescence. . . . .	3
2.1.1. Variable chlorophyll fluorescence. . . . .	3
2.1.2. Chlorophyll fluorescence emission ratio . . . . .	4
2.2. Blue–green fluorescence. . . . .	4
2.3. Epidermal transmittance to UV light. . . . .	5
3. Fluorescence and crop N status. . . . .	5
3.1. Chlorophyll fluorescence. . . . .	5
3.1.1. N–induced changes and applications of variable ChlF. . . . .	5
3.1.2. N–induced changes and applications of the ChlF emission ratio. . . . .	6
3.1.3. N–induced changes and applications of sun–induced ChlF. . . . .	6
3.1.4. Limitations. . . . .	6
3.2. Blue–green fluorescence. . . . .	6
3.2.1. N–induced changes and applications. . . . .	6
3.2.2. Limitations. . . . .	7
3.3. Epidermal transmittance of UV light. . . . .	7
3.3.1. N–induced changes and applications. . . . .	7
3.3.2. Limitations. . . . .	8
4. Fluorescence technologies and precision N applications. . . . .	8
5. Conclusion. . . . .	10
6. Acknowledgements. . . . .	10
7. References. . . . .	10

## 1 Introduction

Plants absorb radiation and use it in photosynthesis, but there is an excess amount of absorbed energy that must be dissipated (Lichtenthaler 1996; Norikane and Kurata 2001). On average, about 75% of visible irradiance is absorbed by leaves, but a very small fraction is converted to organic matter (Fig. 1).



**Fig. 1** Energy budget of a typical leaf. PAR photosynthetically active radiation. Indole blue-stained living section of a wheat flag leaf (courtesy of Corrado Tani and Giovanni Agati)

When a chlorophyll (Chl) molecule absorbs the energy of a given wavelength, a part of it is dissipated by light emission at longer wavelengths within a very short time. This small but variable amount of energy loss is known as the fluorescence emission. Remote sensing of plant characteristics is the evaluation of the partitioning of excitation energy between photochemistry, fluorescence, and thermal dissipation.

Plant stress modifies the relative proportions of absorbed light energy that are used for photosynthetic quantum conversion, chlorophyll fluorescence (ChlF), and heat emission. Stress also modifies the energy flow of photons through the leaf, thereby altering their absorption, reflectance and transmittance properties, and blue-green fluorescence (BGF).

Nitrogen (N) fertilization is the object of intense research efforts around the world. The goal is not only to maintain crop productivity through N recommendations that will generate maximum profits for the farmer but also to protect the environment. Excess N fertilization of crops often leads to a reduction in net returns and groundwater contamination due to  $\text{NO}_3\text{-N}$  leaching (Ferguson et al. 2002; Hashimoto et al. 2007). A balance between supply and utilization is required not only to optimize crop growth and economic returns but minimize the environmental impact as well. Samborski et al. (2009) recently reviewed the strategies available for using plant sensor-based diagnostic information for N recommendations. While the authors mention the existence of satellite or aircraft-based systems, the discussion centers on the most common hand-held and tractor-mounted systems. Only one fluorescence-based device, the hand-held Dualex, is discussed in their paper. The authors describe the need for further research, including work on the combined use of reflectance and fluorescence data for the estimation of plant N status.

Plant metabolism changes quickly (sub-seconds) or more slowly (days, week) in response to environmental changes, but the sensitivity of reflectance-based parameters alone does not provide complete satisfaction. For example, the widely used normalized difference vegetation index (Rouse et al. 1973), which is based on spectral reflectance in the near-infrared and red wavebands, is sensitive to variations in plant growth only at low levels of Chl, low values of leaf area index, and low vegetation cover fractions (Maier and Günther 2001). Tremblay (2004) suggested that: (1) reflectance-based measurements indicate the long-term effects of stress in crop canopies, resulting in variations of Chl content and percent cover of green leaf biomass; (2) Chl content can be expected to change rather slowly (even more slowly than tissue N content, as confirmed by Tartachnyk and Rademacher 2003) in response to most environmental changes and is not affected solely by stresses; (3) fluorescence-based techniques, such as the Dualex, represent innovations in the study of N limitations because they do not rely on the estimation of Chl or biomass status. Canopy fluorescence emissions are associated with biophysical attributes of crop growth that could potentially

assist in the site-specific management of variable rate N fertilization programs (Corp et al. 1998, 2000, 2003).

Chlorophyll fluorescence and UV-excited BGF can be used for the noninvasive detection of stress as they change long before damage of the photosynthetic apparatus is detectable (Lichtenthaler 1996; Colls and Hall 2004; Hura et al. 2006; Lenk et al. 2007; Georgieva et al. 2007). For an overview of aspects related to vegetation stress fluorescence sensing, see Lichtenthaler and Miehé (1997), Buschmann et al. 2000, and Baker and Rosenqvist (2004).

With the recent introduction of commercial devices such as the Miniveg N<sup>TM</sup>, the Dualex<sup>®</sup>, and the Multiplex<sup>®</sup>, there is a need to review the potential and limitations of fluorescence-based techniques for crop N status assessment.

## 2 Plant fluorescence components

### 2.1 Chlorophyll fluorescence

#### 2.1.1 Variable chlorophyll fluorescence

Chlorophyll fluorescence occurs when a photon of light with sufficiently high energy (short wavelengths) is absorbed, exciting an electron within the molecule to a higher energy state (Anderson et al. 1998). Only Chl *a* can participate directly in the light reactions that convert solar energy to chemical energy (Lichtenthaler and Miehé 1997). This characteristic is particularly useful in the study of vegetation (Moya et al. 1992). When all photosynthetic reaction centers are ready to transfer the energy from absorbed radiation along the electron transfer chain, fluorescence is said to be quenched photochemically, which usually occurs when dark-adapted leaves are illuminated. This initial level is called  $F_o$ , attesting that all photosystem II (PSII) reaction centers are open (Fig. 2). In contrast, when reaction centers are fully occupied (reduced, closed), fluorescence is no longer quenched and shows its maximum level ( $F_m$ ), which is about six times higher than its initial level ( $F_o$ ).

$F_v$  (or  $Chl_v$ ) is called the “variable” fluorescence ( $F_v = F_m - F_o$ ), and the parameter  $F_v/F_m$  is referred to as the photochemical efficiency of PSII (Kitajima and Butler 1975) which can reach 0.75–0.85 (Demmig-Adams and Björkman 1987; Bolhar-Nordenkamp et al. 1989; Guidi et al. 2000). It is generally accepted that high  $F_o$  indicates impeded photosynthesis and the presence of stress (Moya et al. 1992).  $F_m$  is measured under saturating irradiance when all of the reaction centers are closed (Baker and Rosenqvist 2004). The ratio  $F_v/F_m$  provides an estimate of the maximum quantum efficiency of PSII photochemistry (Butler 1978) and has been widely used to detect stress-induced perturbations in the photosynthetic apparatus (Baker and Rosenqvist 2004). Similarly, measurement of the light-

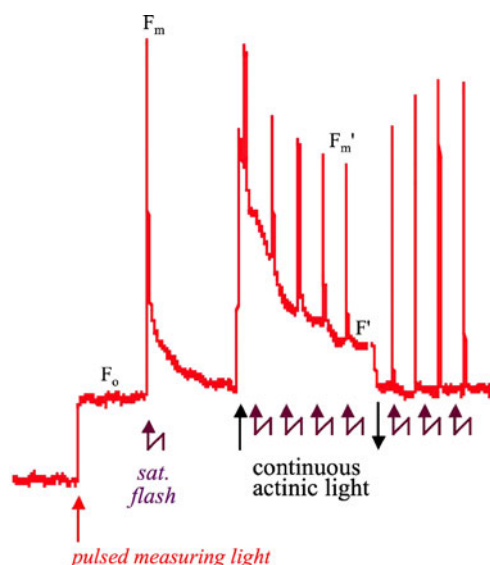


Fig. 2 Variable chlorophyll fluorescence levels

adapted fluorescence signal ( $F'$ ) and the fluorescence signal when all PSII centers are closed under light-adapted conditions ( $F_m'$ ) allows for the estimation of the “operating” photon efficiency of PSII photochemistry, which can be calculated as  $(F_m' - F')/F_m'$  (Genty et al. 1989).

Chlorophyll fluorescence parameters ( $F_o$ ,  $F_v/F_o$ ,  $F_v/F_m$ , non-photochemical quenching, and photochemical quenching) are affected by water stress (Valentini et al. 1994), temperature (Hassan 2006), Chl and flavonoid contents (Lichtenthaler and Miehé 1997; Meyer et al. 2003), pH (Cregg et al. 2004), time of measurement, canopy parameters, and photosynthetic status (Méthy et al. 1994; Günther et al. 1994). The study of these parameter is called quenching analysis (Baker and Rosenqvist 2004), and the related variations of Chl fluorescence are referred to as variable Chl fluorescence, or Kautsky kinetics (Moya et al. 1992). This area of research on Chl fluorescence is characterized by the largest part of the published work by far. Portable ChlF instruments such as the OS-5-FL (Opti-Sciences, Tyngsboro, MA, USA), the LI-COR 6400R (LI-COR, Lincoln, NE, USA), and the pulse amplitude modulation fluorometer (Waltz, Effeltrich, Germany) are widely used to take measurements from individual leaves for the assessment of crop health status (Schreiber and Bilger 1993; Schreiber et al. 1994; Häder et al. 1997; Zarco-Tejada et al. 2002; Adams et al. 1999; Richards et al. 2003; Tartachnyk and Rademacher (2003); Esteban et al. 2010). Nitrogen fertilization can also affect the photosynthetic apparatus and therefore affect variable Chl fluorescence. Yet, attempts to use quenching analyses for N status determination or other agricultural applications are seldom (e.g., Mauromicale et al. 2006; Tartachnyk et al. 2007; Xu et al. 2008) since a fixed distance of measurement is required, which cannot be accommodated in most applica-

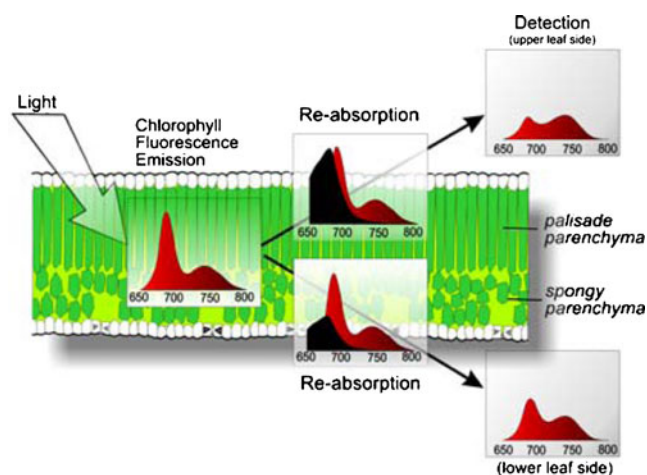
tions. Tartachnyk and Rademacher (2003) suggested that laser-induced fluorescence technologies producing chlorophyll fluorescence emission ratios were more conducive to remote and large-area field measurements as compared with variable chlorophyll fluorescence measurement technologies.

### 2.1.2 Chlorophyll fluorescence emission ratio

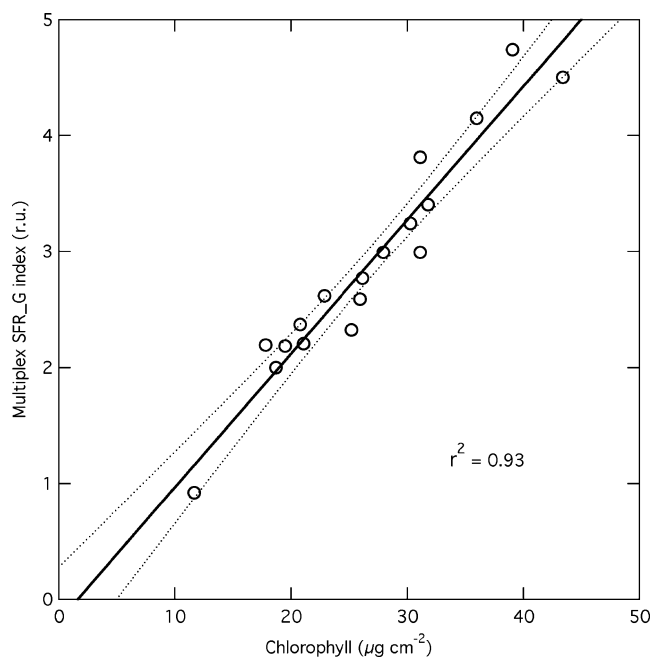
Variation of the Chl fluorescence emission ratio—F685/F730, F690/F730, or F690/F740 (red fluorescence (RF)/far-red fluorescence (FRF); Fig. 3)—associated with stresses can be attributed to either a change in photosynthesis status or a change in the Chl content. This ratio is sensitive to Chl content because of the process of re-absorption of the emitted fluorescence at 690 nm (Agati 1998). In practice, the RF/FRF ratio has mostly been used (Günther et al. 1994; Corp et al. 2003; Apostol et al. 2007; Buschmann 2007; Campbell et al. 2008), but it is sensitive to Chl changes only in lightly green leaves ( $<25 \mu\text{g Chl cm}^{-2}$ ; Moya et al. 1992; Lichtenthaler 1996; Gitelson et al. 1998).

In contrast, the use of the inverse ratio, FRF/RF, varies almost linearly with Chl content in the physiological range of crop leaves ( $20\text{--}60 \mu\text{g Chl cm}^{-2}$ ; Gitelson et al. 1999; Cerovic et al. 2009), and this characteristic makes it the preferred descriptor of the relationship with Chl content. This parameter (renamed SFR) is offered by the recently released Multiplex instrument (Fig. 4).

Because the RF peak has a relatively high contribution to PSII emission (Moya et al. 1992), variable Chl fluorescence (cf. Section 2.1.1) will affect RF peak more than FRF peak



**Fig. 3** Emission and detection of light-induced Chl fluorescence with a scheme indicating the re-absorption of primarily the red Chl fluorescence band by the *in vivo* absorption bands of Chl inside a bifacial leaf. The Chl fluorescence emission spectrum (red) overlaps with the Chl absorption spectrum (black). The re-absorption depends on the Chl density and is higher when detecting the Chl fluorescence from the upper leaf side (high Chl content in the densely packed palisade parenchyma cells) as compared with the lower leaf side (less Chl in the spongy parenchyma layer). With kind permission from Springer Science+Business Media: Buschmann (2007), p. 265, Fig. 3

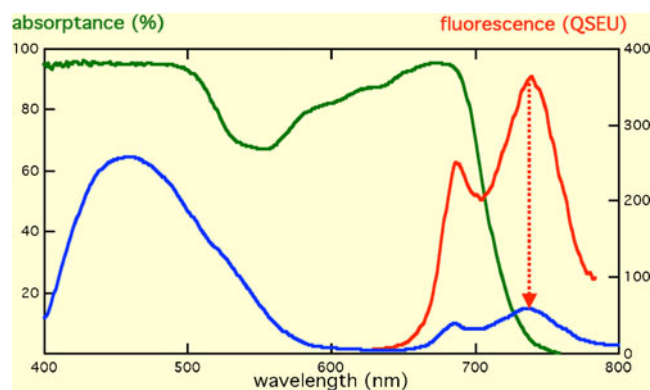


**Fig. 4** Detached Kiwifruit (*Actinidia deliciosa*) leaves of different age measured from the adaxial side. Standard Multiplex configuration: 8-cm diameter illumination surface, 10 cm from the detectors, 1-s measurements, 250 flash average. Chlorophyll estimation by extraction in 100% methanol according to Porra et al. (1989)

so that the RF/FRF ratio also provides a sensitive and noninvasive indicator of environmental stress (Buschmann 2007), but only when no significant reduction in Chl content has occurred (Campbell et al. 2008).

### 2.2 Blue-green fluorescence

Under UV excitation, leaves emit blue fluorescence (BF), green fluorescence (GF), RF, and FRF, with maxima around 450, 530, 685, and 735 nm, respectively (Chappelle et al. 1991; Lang et al. 1992; Cerovic et al. 1999; Ounis et al. 2001; Campbell et al. 2007; Fig. 5).



**Fig. 5** Fluorescence of a leaf in the blue-green and the red–far-red region of the spectrum. The blue line represents BGF and Chl fluorescence produced by a wheat leaf submitted to a UV excitation (355 nm). The red line represents Chl fluorescence obtained under blue (460 nm) excitation

The BF and GF components originate from hydroxycinnamic acids (Chappelle et al. 1991; Lang et al. 1992; Ounis et al. 2001; Meyer et al. 2003). Blue-green fluorescence is primarily emitted by two cinnamic acids, *p*-coumaric acid and ferulic acid (Lichtenthaler and Schweiger 1998; Hura et al. 2007), more specifically, “cell wall-bound” ferulic acid (Morales et al. 1994; Lichtenthaler and Schweiger 1998). Plants that do not have ferulic acid in their cell walls, such as sunflower (*Helianthus annuus* L.), pumpkin (*Cucurbita pepo* L.), and tobacco (*Nicotiana tabacum* L.), exhibit only very faint BGF emissions (Lichtenthaler and Schweiger 1998). Studies have also shown that pyridine nucleotides represent a minor contribution to the BGF of leaves (Lang et al. 1992; Cerovic et al. 1993). In an earlier study, Chappelle et al. (1991) suggested that NADPH was responsible for the BF at 440 nm, but this was the result of a misinterpretation based on indirect correlations with photosynthetic activity. Cerovic et al. (1998) showed that photosynthetic pigments (Chls and carotenoids) reabsorb most of the BF emitted by chloroplast NADPH.

The BGF-emitting components are far more abundant in the epidermis than in the mesophyll, and more on the adaxial side than the abaxial side (Cerovic et al. 1994). Langsdorf et al. (2000) showed that BGF was high in the leaf veins of sugar beet (*Beta vulgaris* L.) plants. Subhash et al. (1999) reported that young yellowish green leaves of an aurea mutant of tobacco showed a much higher BGF emission and lower ChlF as compared with the green wild type. Blue-green fluorescence remains constant over time as long as there are no changes in pigment content or in the optics of the leaf (Buschmann et al. 2000).

### 2.3 Epidermal transmittance to UV light

Nearly all plant leaves accumulate UV-absorbing phenolic compounds (Phen) in their epidermis. Two major classes of UV-screening Phen are known: hydroxycinnamic acids, absorbing predominantly in the UV-B range (280–315 nm), and flavonoids, exhibiting a broader absorbance range including the UV-A (315–400 nm; Cockell and Knowland 1999; Cerovic et al. 2002). Close et al. (2007) reported three classes of Phen compounds (gallotannin, stilbene, and flavonol) that show similar responses to UV-A radiation. Robberecht and Caldwell (1978) found that leaf epidermal transmittance of UV

radiation (280–400 nm) in several plant species was generally <10%. Bilger et al. (1997) reported that sun plants showed lower ratios of UV/BG-induced ChlF, and therefore lower epidermal UV transmittance than shade plants, presumably due to greater UV-screening capacities. Krause et al. (2003) reported that in most cases, UV-A shielding by the abaxial epidermis was lower than shielding by the adaxial epidermis. This is due to the preferential accumulation of flavonols in the adaxial epidermis (Bidel et al. 2007; Fig. 6). Measurements of in vivo ChlF indicated that the ratio of UV-absorbing screening pigments per leaf area increased in monocots from the leaf base to the tip (Wagner et al. 2003; Meyer et al. 2003).

Ultraviolet absorbance of the leaf extracts of tropical forest plants is generally higher in sun leaves than in shade leaves, and the absorbance is much higher in the UV-B spectral region at 305 nm than in the UV-A region at 375 nm (Krause et al. 2003). Stober et al. (1994) reported that plants grown in the field at a high photon flux density showed higher F450/F690 values, primarily caused by a greatly reduced penetration depth of the exciting UV light into the leaf attributable to absorbing substances in the epidermal layers. Nybakken et al. (2004) found that artificially increased UV-B radiation and temperature did not consistently influence epidermal UV-B transmittance in a group of vascular plant species, which suggests that environmental factors other than UV-B radiation may influence epidermal UV-B screening. In monocots, the basal leaf segments that are in the early stage of development are less protected against UV radiation than fully developed mature leaf regions (Wagner et al. 2003). This can even be exploited to characterize leaf developmental stage (Meyer et al. 2003).

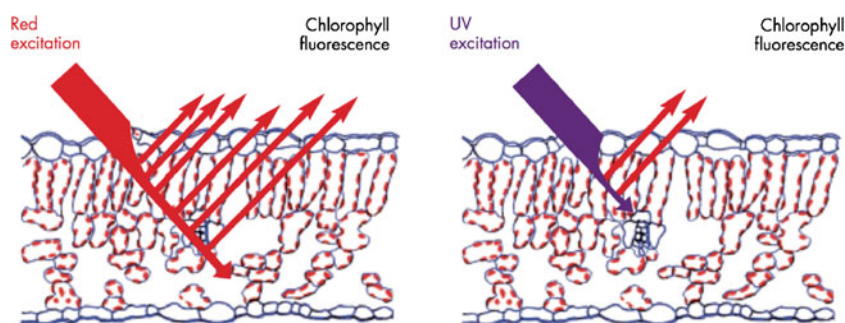
## 3 Fluorescence and crop N status

### 3.1 Chlorophyll fluorescence

#### 3.1.1 N-induced changes and applications of variable ChlF

In a study analyzing the effect of N on ChlFv, Lima et al. (1999) concluded that regardless of external P supply,  $F_v/F_m$  decreased by about 7% and  $F_v/F_o$  by 25% in low-N-content plants of bean (*Phaseolus vulgaris* L.). Huang et al. (2004)

**Fig. 6** Differential screening characteristic of a leaf to UV and red light due to flavonoid accumulation in the adaxial epidermis



reported that there was a higher non-photochemical quenching in N-stressed rice (*Oryza sativa* L.) plants. As N deficiency was maintained,  $F_v/F_m$ ,  $F_v'/F_m'$  (variable/maximum fluorescence during a saturating light flash; Demmig-Adams et al. 1989), and photochemical quenching were lowered more rapidly in N-deficient than in non-deficient plants. These findings are consistent with those reported by Khamis et al. (1990) for corn (*Zea mays* L.) plants. N deficiency may affect PSII photochemistry because N deficiency decreases the quantum yield of PSII electron transport and  $F_v/F_m$  (Lu and Zhang 2000; DaMatta et al. 2002).  $\text{NH}_4^+$  additions produced more complex effects on fluorescence than  $\text{NO}_3^-$  additions (Shelly et al. 2007).

### 3.1.2 N-induced changes and applications of the ChlF emission ratio

Chlorophyll fluorescence emission ratio has now been widely applied to crop N status monitoring. Thanks to the development of specific instruments (cf. Section 4), ChlF has been used to assess N supply, uptake, and biomass under field conditions (Langsdorf et al. 2000; Bredemeier and Schmidhalter 2003; Mauromicale et al. 2006). Thoren and Schmidhalter (2009) reported that N status and aerial biomass dry weight can be determined simultaneously by laser-induced two-wavelength Chl fluorescence measurements with accuracy comparable to conventional methods. Furthermore, the soil does not influence the measured fluorescence signal. This method measures the Chl fluorescence induced by red laser light at 630 nm. The ratio of the 690- and 730-nm fluorescence wavelengths correlates strongly with aboveground N content, with statistical analysis of measurement data providing biomass information (Lejealle et al. 2010; Zhang and Tremblay 2010).

### 3.1.3 N-induced changes and applications of sun-induced ChlF

Sun-induced fluorescence is potentially the best indicator of absorbed photosynthetically active radiation, the most important parameter defining plant productivity (Cerovic et al. 1995; Maier and Günther 2001). However, quantifying the amount of ChlF emitted by a leaf or a canopy under natural sunlight is difficult because the reflected light overwhelms the fluorescence signal. Sun-induced fluorescence (Maier and Günther 2001; Meroni et al. 2010) has been estimated from airborne data (Zarco-Tejada et al. 2009). The 4th International Workshop on Remote Sensing of Vegetation Fluorescence (Valencia, Spain, November 2010) has revealed the current status of this research area (<http://www.congrex.nl/10c23/>). So far, very little of this new application opportunity has addressed the issue of N status assessment. Only contributions from the NASA group (Diurnal and Directional Responses of Chlorophyll Fluores-

cence and the PRI in a Cornfield, by E. Middleton, Y.-B. Cheng, L. Corp, P. Campbell, W. Kustas) reported preliminary results on the R/FR as it related to N pretreatments of corn stands, but in a somewhat inconsistent manner.

### 3.1.4 Limitations

So far, ChlFv has not been widely used to obtain field measurements of N status. Most commercially available fluorimeters proceed from measurements on individual leaves, and screening large numbers of plants is time-consuming (Baker and Rosenqvist 2004). The major limitation for the use of ChlF emission ratio is the larger variations of ChlFv in the RF than in the FRF emission band. Furthermore, some fluorescence parameters, such as F690/F730 and F740/F685, are not very sensitive either to the leaf area and structure of the vegetation or to Chl content (Méthy et al. 1991; Takeuchi et al. 2002). Field measurements of laser-induced two-wavelength Chl fluorescence under variable light conditions showed a linear influence of direct sunlight on the ratio of F690/F730, whereas the ratio was unchanged under diffuse sunlight (Thoren et al. 2010).  $F_v/F_m$  is not considered a robust indicator of nutrient status or relative growth rate (Rodríguez-Román and Iglesias-Prieto 2005; Kruskopf and Flynn 2006).

## 3.2 Blue-green fluorescence

### 3.2.1 N-induced changes and applications

Blue-green fluorescence has been shown to be influenced by the nutrient content in soil (Chappelle et al. 1991). Mercure et al. (2004) stated that the increase in barley (*Hordeum vulgare* L.) leaf Phen compounds observed with limiting N supply was accompanied by large increases of BGF intensity and the BGF/ChlF(UV) ratio of leaf sections. Langsdorf et al. (2000) demonstrated that BGF of sugar beet plants increased at higher N application rates. A similar trend was reported by Heisel et al. (1997) for wheat (*Triticum aestivum* L.), but Campbell et al. (2007) found that BGF decreases in corn with N at high N application rates. For most species and conditions,  $F(\text{UV-B})/F(\text{BG})$  ratios are higher for abaxial than adaxial leaf surfaces (Barnes et al. 2000).

Blue-green fluorescence has been used in conjunction with ChlF to detect plant stress and identify plant types (Chappelle and Williams 1987; Chappelle et al. 1991). Remote sensing of BF appears to be a suitable technique for determining the state of health of terrestrial vegetation (Stober et al. 1994). Images of the fluorescence ratios BF/RF (F440/F690) and BF/FRF (F440/F740) are early stress indicators (Buschmann et al. 2000) and are more sensitive to growth conditions than F690/F740 (Heisel et al. 1996). These two ratios have been used to distinguish sugar beet plants with high N supply from those with low N supply (Langsdorf et al. 2000).

### 3.2.2 Limitations

GF emission intensities excited at 404 nm sometimes were too low to be used (Schweiger et al. 1996). Richards et al. (2003) reported that BF/GF ratios for both bean and wheat plants were insensitive to rapid changes in light levels. Because of the Phen origin of BGF, it is strongly dependent on temperature, like any fluorophore in vitro (Bongi et al. 1994; unlike Chl which is linked to proteins). BGF emission therefore strongly depends on environmental factors experienced by the plant (Chappelle et al. 1984). Leaf BGF increased when leaf temperature decreased (5% of BGF change per degree Celsius), while RF remained unchanged (Bongi et al. 1994; Morales et al. 1998). Therefore, BGF is not a good parameter to use for plant physiological characteristics assessment when temperature is variable. When the temperature does not change during the measurement of the BGF/FRF\_UV ratio, the latter is mostly influenced by the decrease in FRF\_UV due to the screening by epidermal Phen.

### 3.3 Epidermal transmittance of UV light

#### 3.3.1 N-induced changes and applications

The value of leaf UV absorbance is directly correlated with the amount of Phen (Cockell and Knowland 1999; Cerovic et al. 2002). Polyphenolics are mainly present in leaf epidermis and they have typical UV absorption peaks in the UV-A and UV-B (Cerovic et al. 2002). When N is limiting for plant growth, the carbon/nutrient balance hypothesis (Bryant et al. 1983) predicts that carbohydrates will accumulate in plant tissues. The increased content of carbohydrates will lead to increased synthesis of carbon-based secondary metabolites such as Phen and terpenes (Hamilton et al. 2001). An increase in leaf Phen contents has been linked to N deficiency (Cartelat et al. 2005; Tremblay et al. 2007). Lea et al. (2007) showed that N deficiency results in the accumulation of some Phen compounds, such as anthocyanins and flavonols, and in enhanced transcript levels of certain genes important for the accumulation of anthocyanins and flavonols.

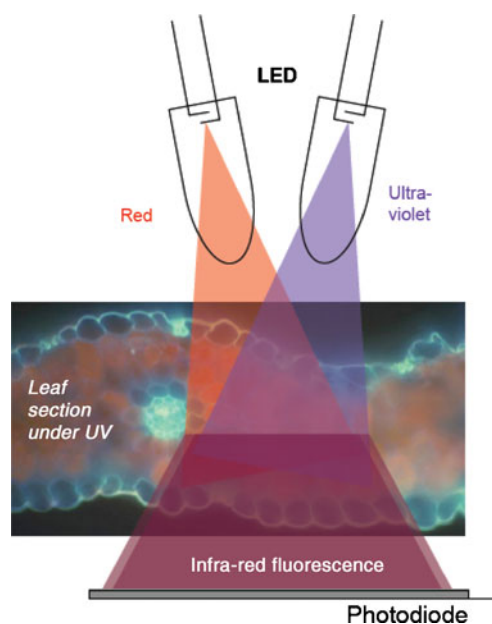
Ounis et al. (2001) showed that the epidermal UV absorption of vegetation can be estimated from the ChlF excitation ratio,  $\Phi F(532)/\Phi F(355)$ . Bilger et al. (1997) and Barnes et al. (2000) reported that noninvasive estimates of epidermal transmittance of UV radiation using ChlF can detect changes in pigmentation and in leaf optical properties induced by UV-B radiation under both field and laboratory conditions. Samson et al. (2000) found that Phen may be a more specific indicator of crop N status than Chl because the latter is sensitive to sulfur deficiency. Bengtsson et al. (2006) stated that ChlF can be used to estimate the content of flavonoids in broccoli (*Brassica*

*oleracea* L.) flower buds by measuring the relative epidermal absorbance of blue light.

Leaf fluorescence emissions significantly increase with N fertilization, reach a plateau (or maximum) for optimal rates, and decline slightly at high N rates (Corp et al. 2003). These changes in UV-induced FRF can be explained by the accumulation of Phen in the epidermis under N deficiency (Cartelat et al. 2005).

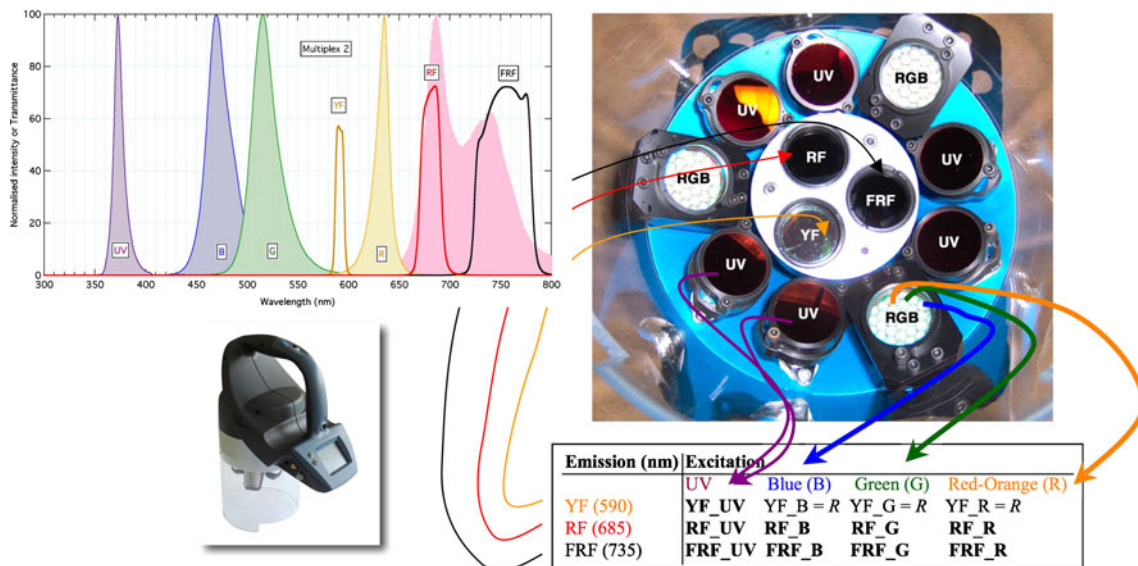
The aforementioned screening of Chl by Phen was used in a commercial leaf clip called Dualex®. This instrument measures UV (375 nm) absorption of the leaf epidermis by the double excitation of ChlF in relation to N availability (Fig. 7). The UV/Visible fluorescence excitation ratio has been described as the most sensitive and consistent parameter in discriminating N treatments (Apostol et al. 2007). The Dualex has been used to assess N status in both wheat and corn (Cartelat et al. 2005; Cerovic et al. 2005; Tremblay et al. 2007; 2010). Dualex was found to have potential for predicting wheat grain protein content (Cerovic et al. 2005).

Multiplex® is the latest hand-held optical fluorescence sensor introduced for the non-destructive measurement of various parameters representative of plant physiological status. The instrument generates fluorescence in the plant tissues using light sources (LED) generating four wavelengths: UV\_A (375 nm, UV); blue (450 nm, B); green (530 nm, G); and red (630 nm, R). Three filtered synchronized detectors record the fluorescence in the following bands: blue-green (447 nm, BGF) when B excitation is not used or yellow (590 nm) when B excitation is used; red (665 nm, RF) and far-red (735 nm, FRF; Fig. 8). The combinations of these excitation and fluorescence signals result in 66 fluorescence ratios potentially useful to interpret the Chl, flavonoid, and N content status of plant



**Fig. 7** Leaf UV epidermal screening estimation. Dualex principle of operation





**Fig. 8** Configuration of the Multiplex instrument in terms of excitation and fluorescence detection wavebands

material. Ratios are preferred over independent signals for their reduced dependency on measurement distance.

Multiplex has potential for in-season assessment of crop N status (Lejealle et al. 2010; Zhang and Tremblay 2010) particularly through the following indices: the Chl index (SFR\_R or SFR\_G under red or green excitation, respectively), the flavonoid index (FLAV), the nitrogen balance index (NBI), the transformed single-fluorescence excitation anthocyanin relative index (FERARI), and the anthocyanins content index (ANTH). SFR is defined as the simple fluorescence emission ratio  $FRF_R/RF_R$  (FRF and RF, respectively, under red excitation). FLAV is defined as  $\log(FRF_R/FRF_{UV})$  (FRF under red and UV excitations, respectively). NBI is the Chl-to-flavonoids ratio computed by the ratio  $FRF_{UV}/RF_R$  (FRF under UV excitation and RF under red excitation). ANTH is computed as the  $\log(FRF_R/FRF_G)$  under red and green excitations. The measurement is made from a 10-cm distance from the light sources, and the measurement surface corresponds to a circle 8 cm in diameter. This may be seen as a limitation particularly in high-throughput systems mounted on carriers where uneven heights in crop stands would call for the device to occasionally touch the leaf, or for sophisticated guiding system. However, a new version of the instrument is being developed that will be able to work from a 20- to 30-cm distance and with a larger depth of view.

### 3.3.2 Limitations

Andersen and Kasperbauer (1971) and Barnes et al. (2000) stated that ChlF was of limited utility for the noninvasive determination of the epidermal UV transmittance of plants in cold environments because UV and low temperatures can

stimulate the production of BG-absorbing pigments that interfere with these indirect measurements of UV transmittance when blue light is used as a reference. This problem is alleviated by using red reference light (Pfundel et al. 2007). In green leaves, epidermal screening of UV radiation works only when the Chl is situated in a layer below the epidermal cell layer in which the flavonoids are located (Bengtsson et al. 2006). Nevertheless, as shown on grape berries (Agati et al. 2007), the screening effect can still be used even when there is overlap between Chl and a flavonoid absorber. In that case, the absorber was anthocyanin and the method was extended also to the screening of visible (green) light by anthocyanins (Ben Ghazlen et al. 2010).

Although not yet adapted for agricultural applications, the potential of fluorescence imaging of crops has also been analyzed. Corp et al. (2003) stated that a fluorescence imaging system and a laser-induced fluorescence imaging system may assist growers in site-specific management of variable rate N fertilization in corn. However, the flash-lamp imaging system, which images red and far-red Chl fluorescence as well as BGF, has much broader potential for use in photosynthesis and ecophysiology research and in plant stress detection (Lichtenthaler et al. 2005).

## 4 Fluorescence technologies and precision N applications

Map- and sensor-based approaches are basic methods of implementing site-specific management for the variable rate application of crop inputs (Bredemeier and Schmidhalter 2003). With the availability of high-quality portable instruments, non-destructive measurements of ChlF can be made

in the field (Blaikie and Chacko 1998), but most fluorescence sensors cannot be used like other on-the-go remote sensors, such as the Yara N Sensor (Yara International ASA, Dülmen, Germany), the GreenSeeker™ RT 200 (NTech Industries Inc., Ukiah, USA), or the Crop Circle ACS-430 Active Crop Canopy Sensor (Holland Scientific, Lincoln, USA). These reflectance-based sensors can be mounted on a tractor and provide information permitting real-time variable rate N application. Time-consuming “dark adaptation” of the plant tissue must be done for quenching analysis (Norikane et al. 2003) and is therefore incompatible with on-the-go sensing. The alternative is to use ChlF emission ratios. Planto GmbH (Leipzig, Germany) has developed a tractor-mounted fluorescence sensor. This sensor can reliably detect N uptake and biomass in wheat and corn fields with a sensed area of 6–7 m<sup>2</sup> (Bredemeier and Schmidhalter 2003). Reflectance and fluorescence methods are sensitive in detecting corn N needs and may be especially powerful in monitoring crop conditions if both types of information can be combined (McMurtrey et al. 2003). The on-the-go information obtained can be coupled with a fertilization algorithm to control the amount of N fertilizer applied (Bredemeier and Schmidhalter 2003). Measurement of leaf ChlF may serve as a pre-visual, early warning indicator of crop N stress (Buschmann et al. 2000). Apostol et al. (2003) demonstrated that fluorescence can discriminate between N-deficient and N-sufficient corn plants. Bélanger et al. (2005) reported that N variables in potato (*Solanum tuberosum* L.) can be detected using ground-based fluorescence; almost 70% of the N-deficient plants and more than 90% of the N-sufficient plants were correctly discriminated. Schächtl et al. (2005) reported that laser-induced fluorescence measurements can be used to estimate the N supply in the wheat canopy and make N recommendations. In general, fluorescence technologies are not used much in precision N management given the limitations on the measurement of fluorescence parameters under natural conditions. Fluorescence, as compared with

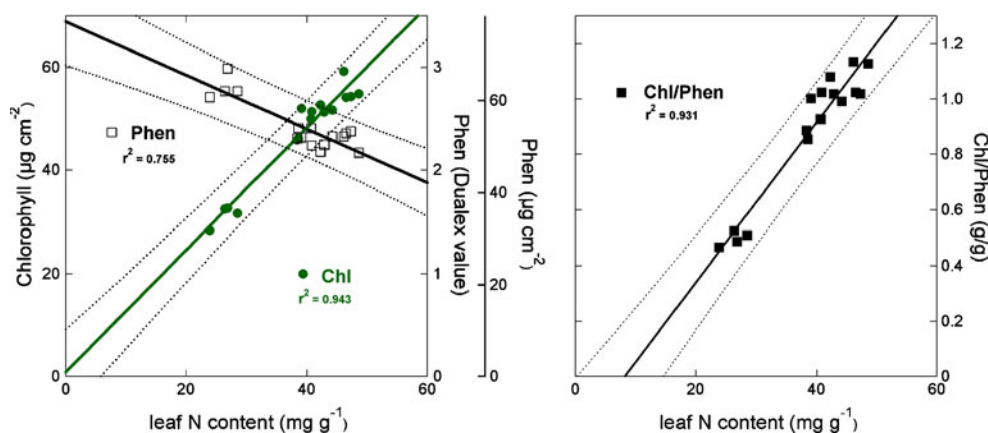
reflectance, eliminates erroneous signals from bare soil, making it more suitable for early growth stages and for widely spaced crops (Heege et al. 2008).

Nowadays, fluorescence-based commercial instruments are available for assessing plant status. Unlike reflectance-based tools, they use approaches that enable N status to be estimated independently from biomass (Thoren and Schmidhalter 2009). Laser-induced fluorescence has also been proposed as a method for discriminating between N-deficient and pathogen-infected winter wheat plants under field conditions (Tartachnyk et al. 2006).

Chlorophyll content increases whereas flavonoids content decreases with increased N application so that NBI increases with N fertility (Fig. 9). The Multiplex is a diagnostic tool that can be used to help N fertilization management remotely and in real time. NBI measured by Multiplex was better correlated with turfgrass leaf N content than Chl content alone (Lejealle et al. 2010). The NBI ratio has been used to map turfgrass N status of a soccer field in time and space. In corn, with the Multiplex, FERARI, ANTH, and FLAV were found to be a particularly sensitive and consistent indicator of N fertility conditions among sampling dates (Zhang and Tremblay 2010). Unlike the chlorophyllmeter, the Multiplex was able to distinguish N treatments equally well in shadow or full sunlight, and at any time during the day.

UV-induced fluorescence can be related to specific variables such as N deficiency and eventually calibrated to provide quantitative recommendations. Under the spectral fluorescent signature principle (or total luminescent spectroscopy (TLS): Dudelzak et al. 1991; Poryvkina et al. 1997), a matrix of fluorescent intensity resulting from several excitation and emission wavelengths is recorded. Total luminescence spectroscopy signatures are represented using 3D graphics with excitation wavelengths on the *y*-axis and fluorescence wavelengths on the *x*-axis. A comprehensive library of signatures is developed out, including both “normal” and “abnormal” cases, which are eventually linked

**Fig. 9** Relationship of Phen and Chl (*left*) and of the Chl/Phen ratio (also called NBI, *right*) with leaf N content for wheat



to a number of typical causes. The potential of this approach for plant canopies has not yet been assessed, but it appears to hold promise. For example, the 12 signals of the Multiplex could be analyzed using TLS.

Cartelat et al. (2005) have shown that Phen content, especially associated with Chl content, is an indicator of wheat N status. Tremblay et al. (2007, 2010) reported that the Chl/Phen ratio was strongly related to the applied dose of N; they found it to be the most sensitive and robust of several indicators throughout the season, for both corn (2007) and wheat (2010). While this Chl/Phen ratio was obtained from combining the measurements of two instruments (a Chl meter and the Dualex 3), it now can be provided by the new Dualex 4 (where it has been relabelled as NBI) in a single operation. Similarly, Multiplex and GreenSeeker devices were recently coupled with a RTK dGPS on a tractor to define variation patterns in vine vigor (Debuisson et al. 2010). These examples of synergy between conventional and fluorescence-based approaches point to the potential of combining such methods in order to obtain a more accurate assessment of crop N status.

## 5 Conclusion

In this paper, the characteristics and applications of four fluorescence sensing approaches—variable chlorophyll fluorescence, leaf chlorophyll content-related fluorescence emission ratio, blue-green fluorescence, and epidermal screening of chlorophyll fluorescence by phenolic compounds—are reviewed from the perspective of crop N status diagnosis. Among all fluorescence techniques, the most important and most exploited signal to date is chlorophyll fluorescence. However, UV-excited blue-green fluorescence has also received a great deal of attention particularly with the recent commercial release of instruments. The use of fluorescence technique in agriculture has still been very limited because fluorescence parameters are undetectable from airborne or mobile platforms. Several studies have shown the considerable potential of combining fluorosensing and multispectral or hyperspectral remote sensing to detect plant nutrient deficiencies (Heisel et al. 1996; Cerovic et al. 1999; Apostol et al. 2003; McMurtrey et al. 2003; Campbell et al. 2007). The combined use of UV-induced fluorescence and hyperspectral imaging has already shown promise for in-field detection of plant stresses (Moshou et al. 2006; Lenk et al. 2007; Chaerle et al. 2007). For the separation of a wide range of N condition levels, hyperspectral (5–10 nm) reflectance indices were found superior as compared with fluorescence or broadband reflectance indices, but they were not able, unlike fluorescence indices, to identify unstressed vegetation conditions (Campbell et al. 2007). Should the interest be confirmed

and the price of the fluorescence-based devices go down, this family of instruments may well constitute a breakthrough in the assessment of crop N fertilization requirements, in line with a better management of this critical and environmentally sensitive production factor. Indeed, fluorescence technology will bring new information that could couple with reflectance remote sensing. The on-the-go information obtained can be valued within a fertilizing algorithm to control the amount of N fertilizer. In the future, the crop diagnosis arsenal will be enriched if this kind of on-the-go sensor system is developed.

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